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New Early Life-Stage Toxicity Test Using the California Grunion (Leuresthes tenuis) and Results with Chlorpyrifos

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16. ABSTRACT

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A New Early Life-Stage Toxicity Test Using the California Grunion (*Leuresthes tenuis*) and Results with Chlorpyrifos[1]

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California grunion were continuously exposed as embryos and fry to technical chlorpyrifos in two toxicity tests conducted in the same exposure apparatus. The first test, a 35-day early life-stage (ELS) test, began with approximately 2.5-day-old embryos that were exposed in flow-through aquaria in darkness until hatching was stimulated on Day 9 of exposure. The second toxicity test, a fry test, began with newly hatched fry and lasted 26 days. Test water temperature ranged from 23 to 26°C and salinity from 24.5 to 34.0‰. Results of the two tests were similar, indicating that exposure of embryos added little to the overall toxicity of chlorpyrifos to grunions. Percentage hatch of embryos was unaffected by the chlorpyrifos concentrations tested. Fry survival was apparently reduced in nominal concentrations ≥ 1.0 μg/liter in both tests, but significantly so in concentrations ≥ 1.0 μg/liter in the ELS test and ≥2.0 μg/liter in the fry test. When compared with carrier controls, mean fish weights were significantly reduced in nominal chlorpyrifos concentrations ≥ 0.5 μg/liter in the ELS test and ≥1.0 μg/liter in the fry exposure. Mean bioconcentration factors were 770× for fish that survived the ELS test and 190× for those that survived the fry test. Results demonstrate the practicality of conducting ELS tests for the first time with a marine fish from the Pacific coastal waters of the United States. © 1985 Academic Press, Inc.

INTRODUCTION

Chronic toxicity tests with fishes are an important component of existing and proposed aquatic hazard assessment procedures (Cairns *et al.*, 1978; Dickson *et al.*, 1979) and are required in derivation of water quality criteria (U.S. EPA, 1980). The sheepshead minnow (*Cyprinodon variegatus*) is the only estuarine fish routinely used in early life-stage (continuous exposure from the embryonic stage to approximately three weeks into the exogenous feeding stage) and life-cycle toxicity tests. Results of chronic tests with sheepshead minnows are frequently similar to those of freshwater fishes (Hansen and Parrish, 1977; Hansen, 1985), but little is known about the chronic sensitivity of other estuarine or marine fishes. To address this need for comparative chronic toxicological data, we have been developing methods and conducting tests with additional species of estuarine fishes (Goodman *et al.*, 1983; Goodman *et al.*, 1985). In this paper, we report results of an early life-stage (ELS) toxicity test and a concurrent fry toxicity test with California grunion (*Leuresthes tenuis*). The ELS test was conducted so that results could be compared directly with those from other such tests in which embryos are continuously

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maintained in water; the fry test was conducted as a more environmentally realistic exposure because California grunion embryos typically develop in damp sand (Walker, 1952) rather than in water and would not be expected to be exposed as embryos to most waterborne pollutants. We believe that the ELS methods we developed are the first such methods suitable for testing a marine fish from the U.S. Pacific Coast.

California grunion are marine atherinids whose principal range is along the California coast from Point Conception to Punta Abreojos (Walker, 1952). They are surface-dwelling fish (Reynolds *et al.,* 1977) that spend most of their lives near the coast and attain a maximum length of approximately 15 to 18 cm (Walker, 1952). Although the spawning season varies, it generally occurs from late February to early September on three or four nights following full or new moons (Walker, 1952). During a few hours immediately after high tide, spawning fish swim as far onto the beach as possible and briefly strand themselves; females partially bury themselves in the sand and release their eggs while males release milt on the sand nearby (Walker, 1952). Eggs are demersal and 1.5 to 1.6 mm in diameter (David, 1939). Embryos typically develop in damp sand for approximately 10 days until the next series of high tides uncover them and hatching occurs (Walker, 1952; Middaugh *et al.,* 1983). California grunion have been reared by several researchers in laboratory studies (David, 1939; Hubbs, 1965; May, 1981; Ehrlich and Farris, 1971, 1972; Reynolds *et al.,* 1976, 1977; Ehrlich and Muszynski, 1982). David (1939) obtained better survival of embryos to hatching in running seawater than in standing water or in sand and observed that only one week of incubation was required at approximately 18 to 20°C. Reynolds *et al.* (1977) determined that 2- to 4-month-old fish exhibited a final modal temperature preferendum of 25°C. Ehrlich and Muszynski (1982) found that yolk-sac larvae preferred approximately 25°C, but maximum larval growth efficiency occurred from 18 to 23°C and that fed larvae beyond the yolk-sac stage and until at least 69 days posthatching preferred temperatures within that range. Ehrlich (1977) and Winkler *et al.* (1983) used California grunion embryos in toxicity tests to determine effects of pollutants on embryonic development and hatching success. However, we are unaware of any chronic toxicity test methods (ELS or life cycle) using this or any other marine fish from the Pacific Coast.

The compound we tested was chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate], a chlorinated organophosphate insecticide used for control of household insects, mosquitoes, fire ants, ornamental and agricultural crop insects, and other purposes (Berg, 1983). In a laboratory study using estuarine water and sediments, its half-life was 24 days (95% confidence interval = 20 to 29) (Schimmel *et al.,* 1983). In flow-through toxicity tests in which chlorpyrifos concentrations were measured, Schimmel *et al.* (1983) derived 96-hr LC_{50} values of 0.035 μg/liter for an estuarine mysid (*Mysidopsis bahia*) and from 1.7 to 136 μg/liter for four species of estuarine fishes. Lower and upper chronic values (highest concentration that caused no significant adverse effect and lowest concentration that caused a significant adverse effect) derived in ELS tests with three estuarine atherinids exposed to technical chlorpyrifos ranged from 0.28 to 0.75 μg/liter and from 0.48 to 1.8 μg/liter, respectively (Goodman *et al.,* 1985). Jarvinen and Tanner (1982) conducted ELS tests with freshwater fathead minnows (*Pimephales promelas*) and derived lower and upper chronic values of 1.6 and 3.2 μg technical chlorpyrifos/

liter and 2.2 and 4.8 μg of a chlorpyrifos slow-release formulation/liter, respectively. In partial life-cycle toxicity tests with fathead minnows exposed to the slow-release chlorpyrifos formulation, the lower chronic value was determined to be <0.12 μg chlorpyrifos/liter (Jarvinen *et al.*, 1983).

MATERIALS AND METHODS

Seawater used in the toxicity tests was pumped from Santa Rosa Sound, Florida, through a sand filter and 20-μm polypropylene filters, and was aerated in a fiberglass reservoir (approximate volume of 9.5 kl, with a residence time of several hours). Water then flowed, as needed, to an aerated constant-head box. A proportional diluter similar to that described by Schimmel *et al.* (1974) delivered 1.0 liter of water to each treatment at each cycle (approximately every 15 min). Stock solutions were prepared with technical chlorpyrifos (purity stated as 97.9% by manufacturer; our analyses indicated 92.0%) and were infused into the diluter mixing boxes with a Hamilton Precision Liquid Dispenser II[3] to provide nominal exposure concentrations of 0.25, 0.50, 1.0, 2.0, and 4.0 μg chlorpyrifos/liter and seawater control and carrier control. Triethylene glycol was used as the carrier solvent at 0.4 μl/liter in the carrier control and all chlorpyrifos treatments. Splitter boxes equipped with two 2-mm-i.d. glass capillary drains delivered the seawater from the diluter to the two adjacent aquaria per treatment. Exposure aquaria (i.d. = 22 cm long × 22 cm wide × 10 cm high) were partially immersed in a freshwater bath that maintained test water temperature at 23 to 26°C. During embryo exposure, the aquaria were equipped with a 5-mm-i.d. self-starting siphon that fluctuated the water depth between 2.0 and 7.5 cm. Two toxicity tests were conducted in the same apparatus.

California grunion embryos were collected on the night of July 9, 1982, at Black's Beach, La Jolla, California. Eggs from approximately 20 to 25 females were stripped into glass culture dishes that contained seawater and fertilized by stripping milt from 5 to 8 males. After 10 min, the eggs were rinsed repeatedly. Water temperature and salinity at the collection site were 19°C and 34 to 35‰. The embryos were shipped by air to our laboratory in glass vacuum bottles filled with seawater and arrived at 25°C and 35‰ salinity. Embryos contained no detectable chlorpyrifos (<0.010 μg/g), other organophosphate pesticides (<0.10 μg/g), polychlorinated biphenyls (<0.10 μg/g), or organochlorine pesticides (<0.20 μg/g). Embryos used in the first toxicity test, a 35-day ELS test, were approximately 2.5 days old, at developmental stages 22 to 24 (Lagler *et al.*, 1977), and had been immersed in seawater and kept in darkness prior to testing. The embryos were examined microscopically and groups of four viable ones were placed randomly in each of 28 incubation cups. This process was repeated until all cups contained 16 embryos. Treatment and duplicate numbers were assigned by using a random number table. Two incubation cups were placed in each of the two duplicate aquaria per treatment. Incubation cups for embryos were constructed by replacing the bottom of a 250-ml glass beaker with 450-μm nylon mesh secured with silicone adhesive. Two 7.5-cm-long glass tubes (8-mm i.d.) attached horizontally on opposite edges supported the cup above the aquarium bottom. Embryos were incubated in darkness until hatching

[3] Hamilton Co., P.O. Box 10030, Reno, Nev. 89510. Mention of trade names or commercial products does not constitute endorsement by the U.S. Environmental Protection Agency.

was stimulated. During this interval, a flashlight was used when taking water samples or checking viability of embryos. On Exposure Day 9 (embryos $\simeq$ 12 days old), embryos were rinsed into fry incubation cups, constructed by attaching 9-cm-high cylinders of 450-μm nylon mesh to the inside walls of 100 mm $\times$ 15 mm glass Petri dish bottoms with silicone adhesive. The embryos were placed on a laboratory shaker and were alternately shaken (approximately 68 cycles/min) and kept stationary for 2-min intervals until no additional fish hatched in two consecutive 2-min periods; two or three intervals of shaking were required. Dead embryos were removed and, after replacing the siphons in the aquaria with 2-mm i.d. siphons that varied the water depth from 5 to 7 cm, the incubation cups were placed in their respective aquaria. The photoperiod was changed from 0L:24D to 12L:12D.

The second toxicity test (fry test) began on Day 10 of the ELS test and lasted 26 days. It was conducted in the same aquaria that contained fry of the ELS test and was terminated one day after the ELS test. Thirteen-day-old embryos that had been incubated in the dark in uncontaminated damp sand at 25°C for approximately 10 days (since arrival at our laboratory) were removed from the sand and immersed in water; the water was stirred to stimulate hatching. Procedures used for distributing newly hatched fry to incubation cups and assignment of concentrations and duplicate numbers were similar to those used for embryos, except that a 100-ml beaker was used to transfer the fry in groups of three or four until each cup contained 15 fry. Two cups were then placed in each of the two aquaria per treatment and exposed alongside the fry hatched from continuously exposed embryos (ELS test).

Fish were fed <24-hr-old *Artemia* nauplii twice daily, except for the last 24 hr of the experiment. Equal volumes of a suspension of nauplii were dispensed to each incubation cup. The suspension was sampled on 7 days and the number of *Artemia* in a volume equal to that dispensed per cup at each feeding averaged 5000 $\pm$ 3600. Dead fry were removed daily and qualitative observations of signs of poisoning were recorded.

At termination of the experiments, fish were killed by immersion in hot water, drained on paper towels, individually weighed, and frozen for subsequent chlorpyrifos analysis. Analytical methods for determining chlorpyrifos concentrations in whole fish and in weekly water samples were as described by Schimmel *et al.* (1983). Recovery of spiked samples for quality assurance of chemical analyses averaged 94% (n = 8) for water and was 100% (n = 1) for tissue. Water concentrations were not adjusted for percentage recovery. Mean measured chlorpyrifos concentrations in the two tests differed slightly (Tables 1 and 2) because the values used in computing the means for the fry test (n = 4 per treatment) were a subset of those from the ELS test (n = 6 per treatment). Dissolved oxygen concentrations were determined weekly in each treatment and treatment averages ranged from 5.7 to 5.8 mg/liter (individual samples ranged from 5.6 to 6.0 mg/liter). Mean salinity, obtained by averaging daily maximum and minimum values, was 28.6‰ (range = 24.5 to 34.0) in the ELS test and 29.3‰ (range = 24.5 to 31.5) in the fry toxicity test. Test water pH was measured once during the experiment and ranged across treatments from 7.6 to 7.9. When sampled on Day 3, suspended solids in our dilution water averaged 7.8 $\pm$ 0.3 mg/liter (n = 3).

Survival and growth data were analyzed by one-way analysis of variance. Survival data were arcsin transformed before analyses. Treatment means were compared by

using Duncan's multiple-range test. A significance level of $\alpha = 0.05$ was chosen for all analyses.

RESULTS

Survival of embryos to hatching was unaffected by chlorpyrifos, but survival of fry was significantly reduced. Survival of embryos averaged 91%; treatment means ranged from 83% in the carrier control to 94% in 0.14 μg/liter (Table 1). In the ELS test, fry survival was significantly reduced in chlorpyrifos concentrations ≥0.63 μg/liter but when embryo and fry survival were combined, the effect noted in 0.63 μg/liter was not statistically significant. Mean combined survival (Table 1) in 1.4 μg/liter (12%) and 2.8 μg/liter (22%) were significantly less than in both the carrier control (78%) and the seawater control (82%). In the test that began with newly hatched fry (Table 2), mean survival in 1.3 μg/liter (47%) and 2.8 μg/liter (3%) was significantly less than in both the carrier control (85%) and seawater control (85%). Signs of poisoning exhibited by fish exposed to chlorpyrifos in the two tests were similar. Fish exposed to ≤0.14 μg chlorpyrifos/liter seldom appeared affected. Reduced activity and a darker appearance of at least some of the fish were frequently observed in 0.28 and 0.30 μg/liter and usually observed at ≥0.62 μg/liter. Abnormal lateral flexure of the back and loss of equilibrium were occasionally observed in 0.62 and 0.63 μg/liter and frequently observed in ≥1.3 μg/liter.

Mean weight of fish that survived both tests was significantly reduced by exposure to chlorpyrifos. In the ELS test, the mean wet weight of fish exposed to 0.30 μg chlorpyrifos/liter was significantly less than that of carrier controls and mean weights of fish exposed to ≥0.63 μg/liter were significantly less than those from both control treatments (Table 1). In the test that began with fry, mean wet weights of fish

TABLE 1

SURVIVAL, AVERAGE WET WEIGHTS, RESIDUES, AND BIOCONCENTRATION FACTORS[a] FOR CALIFORNIA GRUNION (*Leuresthes tenuis*) EXPOSED TO CHLORPYRIFOS FOR 35 DAYS AT 23 TO 26°C[b]

Exposure concentration (μg/liter)		Survival (%)			Average weight	Residue	
Nominal	Measured	Embryos	Fry	Combined	(mg)	(μg/g)	BCF
Seawater control	ND[c,d]	93	88	82	29.1	0.016	
Carrier control	ND	83	94	78	33.7	0.024	
0.25	0.14 ± 0.036[e]	94	92	86	32.1	0.15	1000
0.50	0.30 ± 0.061	92	80	74	27.0[f]	0.21	700
1.0	0.63 ± 0.11	91	62[g]	56	22.2[g]	0.39	620
2.0	1.4 ± 0.19	89	14[g]	12[g]	16.4[g]	—	
4.0	2.8 ± 0.48	92	24[g]	22[g]	12.0[g]	—	

[a] BCF = concentration in whole fish ÷ average measured concentration in water.
[b] Exposure began with embryos approximately 2.5 days after fertilization and hatching was stimulated on Day 9 of exposure.
[c] ND = not detectable (<0.015 μg/liter in water, <0.010 μg/g in tissue).
[d] One of the 6 samples contained detectable chlorpyrifos (0.046 μg/liter).
[e] Mean ± SD.
[f] Significantly different ($\alpha = 0.05$) from response of carrier control.
[g] Significantly different ($\alpha = 0.05$) from response of both control treatments.

TABLE 2

SURVIVAL, AVERAGE WET WEIGHTS, RESIDUES, AND BIOCONCENTRATION FACTORS[a] FOR CALIFORNIA GRUNION (*Leuresthes tenuis*) FRY EXPOSED TO CHLORPYRIFOS FOR 26 DAYS AT 23 TO 26°C[b]

Exposure concentration (μg/liter)		Fry survival	Average weight	Residue	
Nominal	Measured	(%)	(mg)	(μg/g)	BCF
Seawater control	ND[c]	85	30.2	ND	
Carrier control	ND	85	30.3	ND	
0.25	0.13 ± 0.045[d]	87	31.1	ND	
0.50	0.28 ± 0.043	83	29.7	0.015	58
1.0	0.62 ± 0.068	72	22.3[e]	0.038	66
2.0	1.3 ± 0.17	47[e]	16.1[e]	0.58	450
4.0	2.8 ± 0.29	3[e]	15.6[f]	—	

[a] BCF = concentration in whole fish ÷ average measured concentration in water.
[b] Exposure began with newly hatched fry obtained from unexposed embryos that had been incubated in damp sand and the exposure was concurrent with the fry portion of the early life-stage toxicity test (see Table 1).
[c] ND = not detectable (<0.015 μg/liter in water, <0.010 μg/g in tissue).
[d] Mean ± SD.
[e] Significantly different ($\alpha = 0.05$) from response of both control treatments.
[f] Excluded from statistical and chemical analyses because of small sample size.

exposed to ≥0.62 μg chlorpyrifos/liter were significantly less than those from both control treatments (Table 2).

Chlorpyrifos was detected in fish surviving both experiments. Average bioconcentration factors (BCF, chlorpyrifos concentration in whole fish ÷ average chlorpyrifos concentration measured in water) were 770 (range = 620 to 1000) for the ELS test (Table 1) and 190 (range = 58 to 450) for the fry exposure (Table 2). Control fish from the ELS experiment contained chlorpyrifos (0.016 and 0.024 μg/g), but none was detected (<0.010 μg/g) in controls from the fry exposure.

DISCUSSION

Although hatching success was good in this and other tests with the California grunion (D. J. Hansen, unpublished data), we were unable in preliminary tests to obtain satisfactory hatching success in treatments that received the carrier solvent even though the maximum solvent concentrations recommended by ASTM (1983) were not exceeded. During these tests, incubation cups consisted of a 9-cm-high cylinder of 450-μm nylon mesh attached to the inside walls of a 100 × 15 mm glass Petri dish bottom and triethylene glycol concentrations were 4 μl/liter. We believe that the incubation period was sufficient to allow microorganisms to utilize the carrier as a substrate, cause fouling of the mesh, and reduce the dissolved oxygen in the microenvironment near the embryo. Reducing the carrier concentration to 0.4 μl/liter and changing the incubation cup to the style reported in this paper eliminated the hatching problem.

When results of the two tests are compared, the 9 days of embryonic exposure in the ELS test apparently caused little, if any, adverse effects on survival or growth beyond those observed in the fry exposure. The range of estimated chronic values

obtained in the two tests, 0.14 and 0.30 μg/liter in the ELS test and 0.28 and 0.62 μg/liter in the fry test, overlapped.

The average BCFs, 770 in the ELS test and 190 in the fry test, are similar to those for other atherinids exposed to chlorpyrifos in ELS tests. Goodman *et al.* (1985) reported average BCFs of 220 for *Menidia beryllina,* 460 for *M. peninsulae,* and probably <420 for *M. menidia* (no detectable chlorpyrifos in tissue). Jarvinen *et al.* (1983) determined an average BCF of approximately 1700 for fathead minnows exposed to a slow-release formulation of chlorpyrifos for 60 days. All of these values are below 5200, the BCF calculated for chlorpyrifos by Schimmel *et al.,* (1983) using log *P* (the logarithm of the octanol:water partition coefficient).

For five fish species tested in ELS tests with chlorpyrifos (this study; Goodman *et al.,* 1985; Jarvinen and Tanner, 1982), estimated chronic values were somewhat similar; however, the acute sensitivities and acute–chronic ratios were more variable (Table 3). The lower and upper estimated chronic values derived for California grunion in the ELS test were 0.14 and 0.30 μg chlorpyrifos/liter, respectively. Similar estimated chronic values were reported by Goodman *et al.* (1985) for three other atherinids exposed to chlorpyrifos in ELS tests. They were 0.75 and 1.8 μg/liter for *M. beryllina,* 0.28 and 0.48 μg/liter for *M. menidia,* and 0.38 and 0.78 μg/liter for *M. peninsulae.* For the four atherinid species tested, geometric means of the estimated chronic values are within a factor of 6, with *L. tenuis* the most sensitive and *M. beryllina* the least sensitive (Table 3). Jarvinen and Tanner (1982) conducted ELS tests with freshwater fathead minnows (*Pimephales promelas*) exposed to technical chlorpyrifos and to a slow-release formulation of chlorpyrifos in separate 32-day tests and derived estimated chronic values of 1.2 and 3.2 μg/liter and 2.2 and 4.8 μg/liter, respectively. An acute–chronic ratio of 6.5 can be calculated for California grunion by dividing the 96-hr LC_{50} of 1.3 μg chlorpyrifos/liter (obtained in a flow-through test with 28- to 32-day-old grunions and measured concentrations, James R. Clark,[4] unpublished data) by the geometric mean of the chronic values from the ELS test. When acute–chronic ratios for the four species of atherinids are compared (Table 3), they are all with a factor of 3. However, acute–chronic ratios calculable from Jarvinen and Tanner's data for fathead minnows (62 for technical chlorpyrifos and 37 for the slow-release formulation) are quite different from those for atherinids because fathead minnows were relatively insensitive in the acute tests (Table 3). Jarvinen *et al.* (1983) conducted a 200-day partial life-cycle test with fathead minnows exposed to a slow-release formulation of chlorpyrifos and calculated an acute–chronic ratio of >1000 after demonstrating adverse effects on growth of second-generation fish at 0.12 μg/liter, their lowest exposure concentration. Since their 200-day test was >20 times more sensitive than the 32-day ELS test of Jarvinen and Tanner (1982) with the same material, this might suggest that our results and those of Goodman *et al.* (1985) for atherinids exposed to technical chlorpyrifos in ELS tests may not be adequate estimates of life-cycle chronic values.

CONCLUSION

These results are the first to demonstrate the practicality of conducting continuous ELS toxicity tests with a marine fish from Pacific coastal waters of the U.S. They

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TABLE 3

COMPARISON OF ACUTE AND CHRONIC SENSITIVITY OF FIVE SPECIES OF FISHES TO CHLORPYRIFOS

Species	Chlorpyrifos formulation	96-hr LC_{50} (μg/liter)	Type of chronic test[a]	Estimated chronic value (μg/liter)[b]	Acute/chronic ratio[c]	References[d]
Leuresthes tenuis	Technical	1.3	Fry	0.42	3.1	Present study; J. R. Clark, unpublished
L. tenuis	Technical	1.3	ELS	0.20	6.5	Present study; J. R. Clark, unpublished
Menidia beryllina	Technical	4.2	ELS	1.16	3.6	Goodman *et al.* (1985); J. R. Clark, unpublished
M. menidia	Technical	1.7	ELS	0.37	4.6	Goodman *et al.* (1985); Schimmel et al., 1983
M. peninsulae	Technical	1.3	ELS	0.54	2.4	Goodman *et al.* (1985); J. R. Clark, unpublished
Pimephales promelas	Technical	140	ELS	2.26	62	Jarvinen and Tanner (1982)
P. promelas	Slow-release	120	ELS	3.25	37	Jarvinen and Tanner (1982)
P. promelas	Slow-release	120	PLC	<0.12	>1000	Jarvinen *et al.* (1983)

[a] ELS, early life-stage; PLC, partial life cycle.
[b] Geometric mean of lower and upper estimated chronic values.
[c] 96-hr LC_{50} ÷ estimated chronic value.
[d] Chronic reference precedes the acute reference.

also demonstrate that the sensitivity of California grunions to chlorpyrifos is similar to that of other atherinids from the Atlantic and Gulf Coasts (Goodman *et al.*, 1985).

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